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Discovery of 3-Formyl-Tyrosine Metabolites from *Pseudoalteromonas tunicata* through Heterologous Expression

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While chemists have long admired the remarkable and useful set of molecules biosynthesized by bacteria, sequenced bacterial genomes, which now appear at the rate of ~20/month, reveal that we actually know shockingly little about the repertoire of bacterially produced small molecules. Cryptic metabolites, molecules whose existence can be inferred from gene sequences but which have never been seen in the laboratory, greatly outnumber known metabolites.[1−3] As a result, the discovery of cryptic molecules in genomes and their production in the laboratory constitute a major research effort. Most studies have focused on the nonribosomal polypeptide synthetase (NRPS), polyketide synthase (PKS), or NRPS-PKS hybrid pathways, as their large size and repeating motifs make them easier to identify and analyze. Smaller pathways employing less familiar biosynthetic strategies are easy to miss in genome annotations, and small molecules made by these pathways are doubly cryptic because they are detected neither in genome annotations nor in the laboratory. These metabolites likely possess new molecular templates and biological activities. This report describes the search that led to the discovery of previously cryptic metabolites that contain an unusual tyrosine-related amino acid (**1** and **2**) from *Pseudoalteromonas tunicata*.

We and others recently noted the ability of ATP-grasp-type ligases—enzymes that catalyze condensation reactions by activating a carboxyl group as a mixed phosphoric acid anhydride in primary metabolism—to catalyze the formation of amide bonds in idiosyncratically biosynthesized small molecules. Known examples include amide bond formation in the posttranslational modification of the ribosomally synthesized molecules microviridin[4,5] and bacilysin[6] and the amide bonds formed between nonproteogenic amino acids in the dapdiamides.[7] Sequence-based searching for homologous ATP-grasp enzymes led to a promising biosynthetic gene cluster in the marine gammaproteobacterium *Pseudoalteromonas tunicata* D2, a model organism for microbial interactions on marine surfaces and a known producer of a variety of antimicrobial and antifouling compounds.[8]

To identify the products of the ATP-grasp containing gene cluster, the predicted operon was PCR amplified from genomic DNA, cloned into an inducible bacterial expression vector (pET-Duet-1), and transformed into *Escherichia coli.* Culture supernatants were fractionated and analyzed for the production of new compounds by LC/MS and comparison to an empty vector control (Figure 1a). Two new clone-specific peaks with molecular ions *m*/*z* 311 and 210 and absorption peaks at 340 nm were observed in aqueous culture extracts. The corresponding compounds were isolated and identified as 3-formyl-L-tyrosine-L-threonine dipeptide (**1**) and 3-formyl-L-tyrosine (**2**) by a combination of 1D and 2D NMR including gCOSY, gHSQC, and gHMBC experiments (see Supporting Information). The molecular formula of **1** was determined to be $C_{14}H_{19}N_2O_6$ by high resolution ESI-QToF-MS with $[M+H]^+$ *m*/*z* 311.1232 (calc. *m*/*z* 311.1243). HMBC and COSY

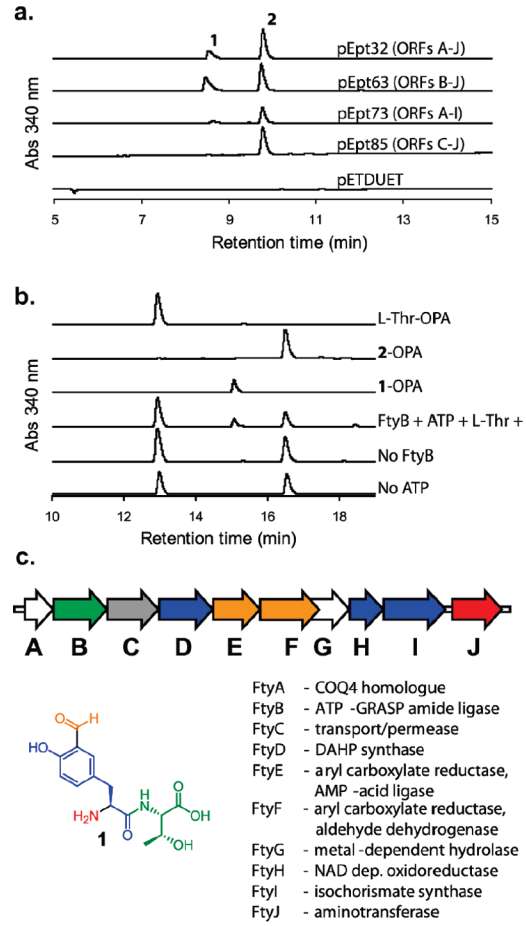
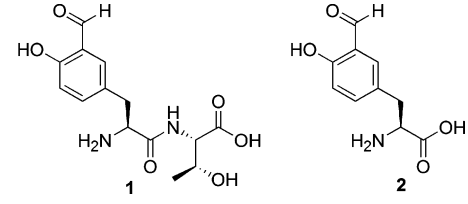


Figure 1. (a) LC/MS traces of heterologous production of **1** and **2**. (b) LC/MS analysis showing ATP-dependent ligation of L-Thr and **2** by FtyB to give **1**. (c) Schematic of 3-formyl-L-tyrosine (*fty*) biosynthetic gene cluster and proposed biosynthesis.

correlations defined the 3-formyl tyrosine as the N-terminal amino acid of dipeptide **1**, and this linkage was verified by the ESI-LCQ-MS/MS ion fragmentation pattern. Optical rotation showed that **2** was the L-enantiomer,[9] and the absolute configuration of **1** was determined by acid hydrolysis and Marfey's derivatization of the resulting amino acids.



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After discovery of these compounds through heterologous expression, we returned to the native organism to find conditions under which **1** and **2** could be produced. We detected **1** and **2** (~1:2 ratio) in aqueous extracts of *P. tunicata* liquid marine broth cultures (see Supporting Information), and their identities were confirmed by LC/MS and comparison to authentic standards (identical retention times, UV−vis spectra, and molecular ion *m*/*z* values).

We initially flagged the *fty* (*formyl tyrosine*) cluster for further study because of the ATP-grasp enzyme FtyB, which is homologous (30% identity) to an ATP-grasp-type amide ligase in dapdiamide biosynthesis (DdaF).[7] When a construct lacking FtyB was expressed in *E. coli* only **2** was observed in culture extracts by LC/MS and extracted ion traces, suggesting FtyB catalyzes amide bond formation between **2** and L-threonine to yield **1** (Figure 1a). To test this hypothesis, a version of FtyB with an N-terminal His_6 tag was cloned, expressed, and purified from *E. coli* (See Supporting Information). FtyB activity was demonstrated using an LC/MS based assay and *o*-phthalaldehyde (OPA) derivatization of substrates and products. FtyB ligates L-Thr and **2** in an ATP-dependent fashion to produce **1** (Figure 1b).

A bioinformatic analysis of the remaining enzymes in the cluster suggests they are involved in the production of **2**, the formylated tyrosine. FtyE and FtyF are closely related to GriC and GriD (39% and 27% identity respectively), which have been shown to function as an aryl carboxylic acid reductase pair in grixazone biosynthesis by *Streptomyces griseus*.[10] FtyE/F could generate the formyl group through conversion of a carboxylic acid to the acyl-AMP intermediate followed by NAD(P)H-dependent reduction. This is a common route to aldehydes in nature; a well-known example is the biosynthesis of the fatty aldehyde substrate of luciferase.[11]

FtyD is homologous to deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (30% identity with class II DAHP synthase from *Streptomyces coelicolor*), the first step in bacterial chorismate biosynthesis. DAHP synthase homologues are commonly present in biosynthetic pathways that rely on the shikimate/chorismate pathway from primary metabolism for substrates as expression of a cluster specific DAHP synthase bypasses the key regulatory step in chorismate production.[12] One possible route to *m*-substituted tyrosine derivatives from chorismate would be through isochorismate and an isoprephenate-like intermediate. Isoprephenate has long been suggested as an intermediate in the production of 3-carboxy-substituted aromatic amino acids in plants but the responsible enzymes have never been identified.[13,14] FtyI contains a 300 residue domain homologous to isochorismate synthase (30%/62% identity/similarity to *E. coli* MenF), and the NAD(P)-dependent oxidoreductase FtyH is a potential isoprephenate dehydrogenase candidate to generate the aromatic ring.

The aminotransferase homologue FtyJ (29% identity, 61% similarity to *Thermus thermophilus* AspAT) likely converts the keto acid to the amino acid. Heterologous expression of a construct lacking FtyJ still afforded **1** and **2**, although in 2-fold lower amounts, which suggests that the activity of FtyJ can be complemented by promiscuous *E. coli* aminotransferases (Figure 1a). FtyA is also not required for heterologous production of **1** and **2**. It has sequence similarity to yeast COQ4 (27% identity), a protein implicated in ubiquinone biosynthesis but with no known enzymatic activity.[15]

While the order of the proposed biosynthetic reactions remains unclear, bioinformatic analysis of the *fty* cluster suggests it contains the necessary elements for constructing 3-formyl-tyrosine. Detailed biochemical investigation of the pathway is under way.

In summary, using a newly discovered role for ATP-grasp enzymes as a search strategy for cryptic metabolites led to the discovery of compounds **1** and **2**. Placing their biosynthetic cluster in an alternative and genetically manipulable host facilitated both the production and detection of these compounds as well as a preliminary interrogation of the individual biosynthetic genes. The particular pathway found, the *Fty* pathway, also indicates that newly discovered biosynthetic clusters will in turn suggest other genes, such as FtyD and the FtyE/F pair, that can serve as the basis for additional searches. A bioinformatic search for clusters containing both FtyE and FtyF homologues revealed >20 unannotated clusters as well as clusters for phenazine (EhpF/G from *Erwinia herbicola*)[16] and thienamycin (ThnN/O from *Streptomyces cattleya*).[17]

Most cryptic metabolites are cryptic because their production is regulated, not constitutive, and their discovery leads to new questions about the regulation of their production and their biological function. In this regard, it is worth noting that compounds **1** and **2** exhibited no antimicrobial activity in agar diffusion assays against *E. coli*, *Bacillus subtilis*, or *Saccromyces cerevisae* at doses up to 20 μg/disk. However, similar synthetic compounds have shown antihypertensive and appetite suppressant activities.[9]

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Supporting Information Available: Experimental details, characterization data for new compounds, and table of sequence homologues. This material is available free of charge via the Internet at http://pubs.acs.org.

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